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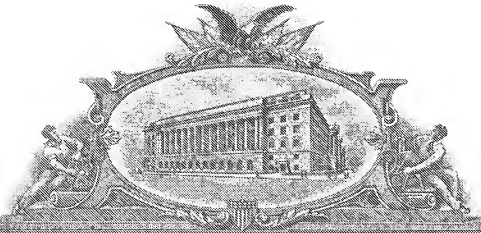

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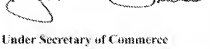

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PROVISIONAL APPLICATION COVER SHEET

Seattle, Washington 98101

January 25, 2005

TO THE COMMISSIONER FOR PATENTS:

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

Transmitted herewith for filing by EXPRESS MAIL is the complete provisional patent application of inventor:

Christopher K. Raymond
Full Name of the Inventor

Seattle, Washington
Residence (City and State or City and Foreign Country)

Title of Invention: METHODS FOR QUANTITATING SMALL RNA MOLECULES

Please address all correspondence to:

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CHRISTENSEN O'CONNOR JOHNSON KINDNESS PLLC
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Seattle, WA 98101

X 1. A provisional application for patent consisting of 17 pages of specification and 1 sheet of drawings is enclosed.

X 2. Transmitted herewith is a sequence listing in printed and computer-readable formats. The paper and computer-readable copies of the sequence listing are the same and do not contain new matter. Entry of the sequence listing into the application is requested.

X 3. Our Check No. 161051 in the amount of $200.00 to cover the total provisional filing fee is enclosed.

X 4. The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16, 1.17, and 1.18 which may be required during the entire pendency of the application, or credit any overpayment, to Deposit Account No. 03-1740. This authorization also hereby includes a request for any extensions of time of the appropriate length required upon the filing of any reply during the entire prosecution of this application. A copy of this document is enclosed.

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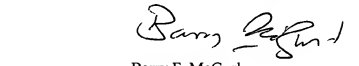
Respectfully submitted,

CHRISTENSEN O'CONNOR
JOHNSON KINDNESS^{PLLC}



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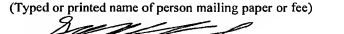

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METHODS FOR QUANTITATING SMALL RNA MOLECULES

FIELD OF THE INVENTION

The present invention relates to methods of amplifying and quantitating small RNA molecules.

BACKGROUND OF THE INVENTION

RNA interference (RNAi) is an evolutionarily conserved process that functions to inhibit gene expression (Bernstein et al. (2001) *Nature* 409:363-6; Dykxhoorn et al. (2003) *Nat. Rev. Mol. Cell. Biol.* 4:457-67). The phenomenon of RNAi was first described in *Caenorhabditis elegans*, where injection of double-stranded RNA (dsRNA) led to efficient sequence-specific gene silencing of the mRNA that was complementary to the dsRNA (Fire et al. (1998) *Nature* 391:806-11). RNAi has also been described in plants as a phenomenon called post-transcriptional gene silencing (PTGS), which is likely used as a viral defense mechanism (Jorgensen (1990) *Trends Biotechnol.* 8:340-4; Brigneti et al. (1998) *EMBO J.* 17:6739-46; Hamilton & Baulcombe (1999) *Science* 286:950-2).

An early indication that the molecules that regulate PTGS were short RNAs processed from longer dsRNA was the identification of short 21 to 22 nucleotide dsRNA derived from the longer dsRNA in plants (Hamilton & Baulcombe (1999) *Science* 286:950-2). This observation was repeated in *Drosophila* embryo extracts where long dsRNA was found processed into 21-25 nucleotide short RNA by the RNase III type enzyme, Dicer (Elbashir et al. (2001) *Nature* 411:494-8; Elbashir et al. (2001) *EMBO J.* 20:6877-88; Elbashir et al. (2001) *Genes Dev.* 15:188-200). These observations led Elbashir et al to test if synthetic 21-25 nucleotide synthetic dsRNAs function to specifically inhibit gene expression in *Drosophila* embryo lysates and mammalian cell

culture (Elbashir et al. (2001) *Nature* 411:494-8; Elbashir et al. (2001) *EMBO J.* 20:6877-88; Elbashir et al. (2001) *Genes Dev.* 15:188-200). They demonstrated that small interfering RNAs (siRNAs) had the ability to specifically inhibit gene expression in mammalian cell culture without induction of the interferon response.

These observations led to the development of techniques for the reduction, or elimination, of expression of specific genes in mammalian cell culture, such as plasmid-based systems that generate hairpin siRNAs (Brummelkamp et al. (2002) *Science* 296:550-3; Paddison et al. (2002) *Genes Dev.* 16:948-58; Paddison et al. (2002) *Proc. Natl. Acad. Sci. U.S.A.* 99:1443-8; Paul et al. 2002) *Nat. Biotechnol.* 20:404-8). siRNA molecules can also be introduced into cells, *in vivo*, to inhibit the expression of specific proteins (see, e.g., Soutschek, J., et al., *Nature* 432 (7014): 173-178 (2004)).

siRNA molecules have promise both as therapeutic agents for inhibiting the expression of specific proteins, and as targets for drugs that affect the activity of siRNA molecules that function to regulate the expression of proteins involved in a disease state. A first step in developing such therapeutic agents is to measure the amounts of specific siRNA molecules in different cell types within an organism, and thereby construct an "atlas" of siRNA expression within the body. Additionally, it will be useful to measure changes in the amount of specific siRNA molecules in specific cell types in response to a defined stimulus, or in a disease state.

Short RNA molecules are difficult to quantitate. For example, with respect to the use of PCR to amplify and measure the small RNA molecules, most PCR primers are longer than the small RNA molecules, and so it is difficult to design a primer that has significant overlap with a small RNA molecule, and that selectively hybridizes to the small RNA molecule at the temperatures used for primer extension and PCR amplification reactions.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides methods for amplifying a microRNA molecule to produce cDNA molecules. The methods include the steps of: (a) using primer extension to make a DNA molecule that is complementary to a template microRNA molecule; and (b) using a forward primer and a reverse primer to amplify the DNA molecule to produce amplified DNA molecules, wherein one of the forward primer and the reverse primer comprises a locked nucleic acid molecule. It will be understood

that, in the practice of the present invention, typically numerous (e.g., millions) of individual microRNA molecules are amplified in a sample (e.g., a solution of RNA molecules isolated from living cells).

In another aspect, the present invention provides methods for measuring the amount of a target microRNA in a multiplicity of different cell types within a living organism. The methods of this aspect of the invention include the step of measuring the amount of a target microRNA molecule in a multiplicity of different cell types within a living organism, wherein the amount of the target microRNA molecule is measured by a method including the steps of: (1) using primer extension to make a DNA molecule complementary to the target microRNA molecule isolated from a cell type of a living organism; (2) using a forward primer and a reverse primer to amplify the DNA molecule to produce amplified DNA molecules, wherein one of the forward primer and the reverse primer comprises a locked nucleic acid molecule; and (3) measuring the amount of the amplified DNA molecules.

The present invention is useful, for example, for quantitating specific microRNA molecules within different types of cells in a living organism, or, for example, for measuring changes in the amount of specific microRNAs in living cells in response to a stimulus (e.g., in response to administration of a drug).

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same become better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIGURE 1 shows a flow chart of a representative method of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

In accordance with the foregoing, in one aspect, the present invention provides methods for amplifying a microRNA molecule to produce cDNA molecules. The methods include the steps of: (a) using primer extension to make a DNA molecule that is complementary to a template microRNA molecule; and (b) using a forward primer and a reverse primer to amplify the DNA molecule to produce amplified DNA molecules, wherein one of the forward primer and the reverse primer comprises a locked nucleic acid molecule.

As used herein, the term "locked nucleic acid molecule" (abbreviated as LNA molecule) refers to a nucleic acid molecule that includes a 2'-O,4'-C-methylene-β-D-ribofuranosyl moiety. Exemplary 2'-O,4'-C-methylene-β-D-ribofuranosyl moieties, and exemplary LNAs including such moieties, are described, for example, in Petersen, M. and Wengel, J., *Trends in Biotechnology 21*(2):74-81 (2003) which publication is incorporated herein by reference in its entirety.

As used herein, the term "microRNA" refers to an RNA molecule that has a length in the range of from 21 nucleotides to 25 nucleotides. Some microRNA molecules (e.g., siRNA molecules) function in living cells to regulate gene expression.

Representative method of the invention. FIGURE 1 shows a flowchart of a representative method of the present invention. In the method represented in FIGURE 1, a microRNA is the template for synthesis of a complementary first DNA molecule. The synthesis of the first DNA molecule is primed by an extension primer, and so the first DNA molecule includes the extension primer and newly synthesized DNA (represented by a dotted line in FIGURE 1). The synthesis of DNA is catalyzed by reverse transcriptase.

The extension primer includes a first portion (abbreviated as FP in FIGURE 1) and a second portion (abbreviated as SP in FIGURE 1). The first portion hybridizes to the microRNA template, and the second portion includes a nucleic acid sequence that hybridizes with the reverse primer, as described *infra*.

A quantitative polymerase chain reaction is used to make a second DNA molecule that is complementary to the first DNA molecule. The synthesis of the second DNA molecule is primed by the reverse primer that has a sequence that is selected to specifically hybridize to a portion of the target first DNA molecule. Thus, the reverse primer does not hybridize to nucleic acid molecules other than the first DNA molecule. The reverse primer includes at least one LNA molecule located within the portion of the reverse primer that does not overlap with the extension primer. In FIGURE 1, the LNA molecules are represented by shaded ovals.

A universal forward primer hybridizes to the 3' end of the second DNA molecule and primes synthesis of a third DNA molecule. It will be understood that, although a single microRNA molecule, single first DNA molecule, single second DNA molecule, single third DNA molecule and single extension, forward and reverse primers are shown

in FIGURE 1, typically the practice of the present invention uses reaction mixtures that include numerous copies (e.g., millions of copies) of each of the foregoing nucleic acid molecules.

The steps of the methods of the present invention are now considered in more detail.

Preparation of microRNA molecules useful as templates. microRNA molecules useful as templates in the methods of the invention can be isolated from any organism (e.g., eukaryote, such as a mammal) or part thereof, including organs, tissues, and/or individual cells (including cultured cells). Any suitable RNA preparation that includes microRNAs can be used, such as total cellular RNA.

RNA may be isolated from cells by procedures that involve lysis of the cells and denaturation of the proteins contained therein. Cells of interest include wild-type cells, drug-exposed wild-type cells, modified cells, and drug-exposed modified cells.

Additional steps may be employed to remove some or all of the DNA. Cell lysis may be accomplished with a nonionic detergent, followed by microcentrifugation to remove the nuclei and hence the bulk of the cellular DNA. In one embodiment, RNA is extracted from cells of the various types of interest using guanidinium thiocyanate lysis followed by CsCl centrifugation to separate the RNA from DNA (see, Chirgwin et al., 1979, *Biochemistry 18*:5294-5299). Separation of RNA from DNA can also be accomplished by organic extraction, for example, with hot phenol or phenol/chloroform/isoamyl alcohol.

If desired, RNase inhibitors may be added to the lysis buffer. Likewise, for certain cell types, it may be desirable to add a protein denaturation/digestion step to the protocol.

The sample of RNA can comprise a multiplicity of different microRNA molecules, each different microRNA molecule having a different nucleotide sequence. In a specific embodiment, the microRNA molecules in the RNA sample comprise at least 100 different nucleotide sequences. In other embodiments, the microRNA molecules of the RNA sample comprise at least 500, 1,000, 5,000, 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000 90,000, or 100,000 different nucleotide sequences.

Synthesis of DNA molecules using microRNA molecules as templates. In the practice of the methods of the invention, first DNA molecules are synthesized that are

complementary to the microRNA template molecules, and that are composed of an extension primer and newly synthesized DNA (wherein the extension primer primes the synthesis of the newly synthesized DNA). Individual first DNA molecules can be complementary to a whole microRNA template molecule, or to a portion thereof; although typically an individual first DNA molecule is complementary to a whole microRNA template molecule. Thus, in the practice of the methods of the invention, a population of first DNA molecules is synthesized that includes individual DNA molecules that are each complementary to all, or to a portion, of a template microRNA molecule.

Any reverse transcriptase molecule can be used to synthesize the first DNA molecules, such as those derived from Moloney murine leukemia virus (MMLV-RT), avian myeloblastosis virus (AMV-RT), bovine leukemia virus (BLV-RT), Rous sarcoma virus (RSV) and human immunodeficiency virus (HIV-RT). A reverse transcriptase lacking RNaseH activity (e.g., SUPERSCRIPT III™ sold by Invitrogen, 1600 Faraday Avenue, PO Box 6482, Carlsbad, California 92008) is preferred in order to minimize the amount of double-stranded cDNA synthesized at this stage. The reverse transcriptase molecule should also preferably be thermostable so that the DNA synthesis reaction can be conducted at as high a temperature as possible, while still permitting hybridization of primer to the microRNA template molecules.

Priming the synthesis of the first DNA molecules. The synthesis of the first DNA molecules is primed using an extension primer. Typically, the length of the extension primer is in the range of from 10 nucleotides to 100 nucleotides, such as 30 to 35 nucleotides. The nucleic acid sequence of the extension primer is incorporated into the sequence of each, synthesized, DNA molecule. The extension primer includes a first portion that hybridizes to a portion of the microRNA molecule. Typically the first portion of the extension primer includes the 3'-end of the extension primer. The first portion of the extension primer typically has a length in the range of from 6 nucleotides to 20 nucleotides, such as from 10 nucleotides to 12 nucleotides.

The extension primer also includes a second portion that typically has a length of from 18 to 25 nucleotides. For example, the second portion of the extension primer can be 20 nucleotides long. The second portion of the extension primer is located 5' to the first portion of the extension primer, and is typically contiguous with the first portion of

the extension primer. The second portion of the extension primer includes at least a portion of the hybridization site for the forward primer. For example, the second portion of the extension primer can include all of the hybridization site for the forward primer, or, for example, can include as little as a single nucleotide of the hybridization site for the forward primer (the remaining portion of the hybridization site for the forward primer can, for example, be located in the first portion of the extension primer). An exemplary nucleic acid sequence of a second portion of an extension primer is 5' CATGATCAGCTGGGCCAAGA 3" (SEQ ID NO:1).

Amplification of the DNA molecules. In the practice of the methods of the invention, the DNA molecules are enzymatically amplified using the polymerase chain reaction. A forward primer and a reverse primer are used to prime the polymerase chain reaction. The reverse primer includes a nucleic acid sequence that is selected to specifically hybridize to a portion of a first DNA molecule.

The reverse primer typically has a length in the range of from 12 nucleotides to 20 nucleotides. The reverse primer hybridizes to the first DNA molecule. The nucleotide sequence of the reverse primer is selected to hybridize to a specific target nucleotide sequence under defined hybridization conditions. The reverse primer and extension primer are both present in the PCR reaction mixture, and so the reverse primer should be sufficiently long so that the melting temperature (Tm) is at least 50°C, but should not be so long that there is extensive overlap with the extension primer which may cause the formation of "primer dimers." "Primer dimers" are formed when the reverse primer hybridizes to the extension primer, and uses the extension primer as a substrate for DNA synthesis, and the extension primer hybridizes to the reverse primer, and uses the reverse primer as a substrate for DNA synthesis. To avoid the formation of "primer dimers," typically the reverse primer and the extension primer are designed so that they do not overlap with each other by more than 6 nucleotides. If it is not possible to make a reverse primer having a Tm of at least 50°C, and wherein the reverse primer and the extension primer do not overlap by more than 6 nucleotides, then it is preferable to lengthen the reverse primer (since Tm usually increases with increasing oligonucleotide length) and decrease the length of the extension primer.

The reverse primer primes the synthesis of a second DNA molecule that is complementary to the first DNA molecule. The forward primer hybridizes to the portion

of the second DNA molecule that is complementary to the second portion of the extension primer which is incorporated into all of the first DNA molecules. The forward primer primes the synthesis of third DNA molecules. The forward primer typically has a length in the range of from 16 nucleotides to 30 nucleotides. The forward primer may include at least one locked nucleic acid molecule. In some embodiments, the forward primer includes from 1 to 3 locked nucleic acid molecules. The nucleic acid sequence of an exemplary forward primer is set forth in SEQ ID NO:2.

In general, the greater the number of amplification cycles during the polymerase chain reaction, the greater the amount of amplified DNA that is obtained. On the other hand, too many amplification cycles (e.g., more than 35 amplification cycles) may result in spurious and unintended amplification of non-target double-stranded DNA. Thus, in some embodiments, a desirable number of amplification cycles is between one and 45 amplification cycles, such as from one to 25 amplification cycles, or such as from five to 15 amplification cycles, or such as ten amplification cycles.

Use of LNA molecules and selection of primer hybridization conditions: hybridization conditions are selected that promote the specific hybridization of a primer molecule to the complementary sequence on a substrate molecule. With respect to the hybridization of a 12 nucleotide first portion of an extension primer to a microRNA, it has been found that specific hybridization occurs at a temperature of 50°C. Similarly, it has been found that hybridization of a 20 nucleotide forward primer to a complementary DNA molecule, and hybridization of a reverse primer (having a length in the range of from 12-20 nucleotides) to a complementary DNA molecule occurs at a temperature of 50°C. By way of example, it is often desirable to design extension, reverse and forward primers that each have a hybridization temperature in the range of from 50°C to 60°C.

LNA molecules can be incorporated into at least one of the extension primer, reverse primer, and forward primer to raise the Tm of one, or more, of the foregoing primers to at least 50°C. Incorporation of an LNA molecule into the portion of the reverse primer that hybridizes to the template microRNA, but not to the extension primer, is typically required because this portion of the reverse primer is typically no more than 10 nucleotides in length. For example, the portion of the reverse primer that hybridizes to the template microRNA, but not to the extension primer, may include at least one locked nucleic acid molecule (e.g., from 1 to 3 locked nucleic acid molecules).

The number of LNA residues that must be incorporated into a specific primer to raise the Tm to a desired temperature mainly depends on the length of the primer and the nucleotide composition of the primer. A tool for determining the effect on Tm of one or more LNAs in a primer is available on the Internet Web site of Exiqon, Bygstubben 9, DK-2950 Vedbæk, Denmark.

Although one or more LNAs can be included in any of the primers used in the practice of the present invention, it has been found that the efficiency of synthesis of cDNA is low if an LNA is incorporated into the extension primer. While not wishing to be bound by theory, LNAs may inhibit the activity of reverse transcriptase.

Detecting and measuring the amount of the amplified DNA molecules: the amplified DNA molecules can be detected and quantitated by the presence of detectable marker molecules, such as fluorescent molecules. For example, the amplified DNA molecules can be detected and quantitated by the presence of a dye (e.g., SYBR green) that preferentially or exclusively binds to double stranded DNA during the PCR amplification step of the methods of the present invention. For example, Molecular Probes, Inc. (29851 Willow Creek Road, Eugene, OR 97402) sells quantitative PCR reaction mixtures that include SYBR green dye. By way of further example, another dye (referred to as "BEBO") that can be used to label double stranded DNA produced during real-time PCR is described by Bengtsson, M., et al., *Nucleic Acids Research 31*(8):e45 (April 15,2003), which publication is incorporated herein by reference. Again by way of example, a forward and/or reverse primer that includes a fluorophore and quencher can be used to prime the PCR amplification step of the methods of the present invention. The physical separation of the fluorophore and quencher that occurs after extension of the labeled primer during PCR permits the fluorophore to fluoresce, and the fluorescence can be used to measure the amount of the PCR amplification products. Examples of commercially available primers that include a fluorophore and quencher include Scorpion primers and Uniprimers, which are both sold by Molecular Probes, Inc.

Representative uses of the present invention: the present invention is useful for producing cDNA molecules from microRNA template molecules. The amount of the DNA molecules can be measured which provides a measurement of the amount of template microRNA molecules in the starting material. For example, the methods of the present invention can be used to measure the amount of specific microRNA molecules

(e.g., specific siRNA molecules) in living cells. Again by way of example, the present invention can be used to measure the amount of specific microRNA molecules (e.g., specific siRNA molecules) in different cell types in a living body, thereby producing an "atlas" of the distribution of specific microRNA molecules within the body. Again by way of example, the present invention can be used to measure changes in the amount of specific microRNA molecules (e.g., specific siRNA molecules) in response to a stimulus, such as in response to treatment of a population of living cells with a drug.

Thus, in another aspect, the present invention provides methods for measuring the amount of a target microRNA in a multiplicity of different cell types within a living organism (e.g., to make a microRNA "atlas" of the organism). The methods of this aspect of the invention each include the step of measuring the amount of a target microRNA molecule in a multiplicity of different cell types within a living organism, wherein the amount of the target microRNA molecule is measured by a method comprising the steps of: (1) using primer extension to make a DNA molecule complementary to the target microRNA molecule isolated from a cell type of a living organism; (2) using a forward primer and a reverse primer to amplify the DNA molecule to produce amplified DNA molecules, wherein one of the forward primer and the reverse primer comprises a locked nucleic acid molecule; and (3) measuring the amount of the amplified DNA molecules. The measured amounts of amplified DNA molecules can, for example, be stored in an interrogatable database in electronic form, such as on a computer-readable medium (e.g., a floppy disc).

The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention.

EXAMPLE 1

This Example describes a representative method of the invention for producing DNA molecules from microRNA template molecules.

Primer extension was conducted as follows (using InVitrogen SuperScript III® reverse transcriptase and following the guidelines that were provided with the enzyme). The following reaction mixture was prepared on ice:

1 μl of 10 mM dNTPs

1 μl of 2 μM extension primer

1- 5 μl of template

4 µl of "5X cDNA buffer"

1 µl of 0.1 M DTT

1 µl of RNAse OUT

1 µl of SuperScript III® enzyme

water to 20 µl

The mixture was incubated at 50°C for 30 minutes, then 85°C for 5 minutes, then cooled to room temperature and diluted 10-fold with TE (10 mM Tris, pH 7.6, 0.1 mM EDTA).

Real-time PCR was conducted using an ABI 7900 HTS detection system (Applied Biosystems, Foster City, California, U.S.A.) by monitoring SYBR® green fluorescence of double-stranded PCR amplicons as a function of PCR cycle number. A typical 10 µl PCR reaction mixture contained:

5 µl of 2X SYBR® green master mix (ABI)

0.8 µl of 10 µM universal forward primer

0.8 µl of 10 µM reverse primer

1.4 µl of water

2.0 µl of template (10-fold diluted RT reaction).

The reaction was monitored through 40 cycles of standard "two cycle" PCR (95°C – 15 sec, 60°C – 60 sec) and the fluorescence of the PCR products was measured.

The foregoing method was successfully used in eleven primer extension PCR assays for quantitation of endogenous microRNAs present in a sample of total RNA. The DNA sequences of the extension primers, the universal forward primer sequence, and the LNA substituted reverse primers, used in these 11 assays are shown in Table 1.

TABLE 1

Target microRNA	Primer number	Primer Name	DNA sequence (5' to 3')	SEQ ID NO
gene-specific extension primers[1]				
humanb let7a	357	let7aP4	*CATGATCAGCTGGGCCAAGA*AACTATACAACCT	2
human miR-1	337	miR1P5	*CATGATCAGCTGGGCCAAGA*TACATACTTCT	3
human miR-15a	344	miR15aP3	*CATGATCAGCTGGGCCAAGA*CACAAACCATTATG	4
human miR-16	351	miR16P2	*CATGATCAGCTGGGCCAAGA*CGCCAATATTTACGT	5

Target microRNA	Primer number	Primer Name	DNA sequence (5' to 3')	SEQ ID NO
human miR-21	342	miR21P6	*CATGATCAGCTGGGCCAAGA*TCAACATCAGT	6
human miR-24	350	miR24P5	*CATGATCAGCTGGGCCAAGA*CTGTTCCTGCTG	7
human miR-122	222	122-E5F	*CATGATCAGCTGGGCCAAGA*ACAAACACCATTGTCA	8
human miR-124	226	124-E5F	*CATGATCAGCTGGGCCAAGA*TGGCATTCACCGCGTG	9
human miR-143	362	miR143P5	*CATGATCAGCTGGGCCAAGA*TGAGCTACAGTG	10
human miR-145	305	miR145P2	*CATGATCAGCTGGGCCAAGA*AAGGGATTCCTGGGAA	11
human miR-155	367	miR155P3	*CATGATCAGCTGGGCCAAGA*CCCCTATCACGAT	12
[1] - Universal forward primer binding sites are shown in italics. The overlap with the RNA-specific reverse primers are underlined.				
universal forward primer				
	230	E5F	CATGATCAGCTGGGCCAAGA	13
RNA species-specific reverse primers[2]				
human let7a	290	miRlet7a-1,2,3R	TGAGGTAGTAGGTTG	14
human miR-1	285	miR1-1,2R	TGGAATGTAAAGAAGTA	15
human miR-15a	287	miR15aR	TAGCAGCACATAATG	16
human miR-16	289	miR16-1,2R	TAGCAGCACGTAAA	17
human miR-21	286	miR21R	TAGCTTATCAGACTGAT	18
human miR-24	288	miR24-1,2R	TGGCTCAGTTCAGC	19
human miR-122	234	122LNAR	TGGAGTGTGACAA	20
human miR-124	235	124LNAR	TTAAGGCACGCG	21
human miR-143	291	miR143R	TGAGATGAAGCACTG	22
human miR-145	314	miR145R2	GTCCAGTTTTCCCA	23
human miR-155	293	miR155R	TTAATGCTAATCGTGA	24
[2] - LNA molecules are shown in bold font. Region of overlap of the reverse primers with the corresponding extension primers are underlined.				

The assay was capable of detecting microRNA in a concentration range of from 2 nM to 20 fM. The assays were linear at least up to a concentration of 2 nM of synthetic microRNA (>1,000,000 copies/cell).

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for amplifying a microRNA molecule to produce DNA molecules, the method comprising the steps of:

(a) using primer extension to make a DNA molecule that is complementary to a template microRNA molecule; and

(b) using a forward primer and a reverse primer to amplify the DNA molecule to produce amplified DNA molecules, wherein one of the forward primer and the reverse primer comprises a locked nucleic acid molecule.

2. A method of Claim 1 wherein the primer extension uses an extension primer having a length in the range of from 10 to 100 nucleotides.

3. A method of Claim 1 wherein the primer extension uses an extension primer having a length in the range of from 30 to 35 nucleotides.

4. A method of Claim 1 wherein the extension primer comprises a first portion that hybridizes to a portion of the microRNA molecule.

5. A method of Claim 4 wherein the first portion has a length in the range of from 10 to 12 nucleotides.

6. A method of Claim 4 wherein the extension primer comprises a second portion.

7. A method of Claim 6 wherein the second portion has a length of from 18 to 25 nucleotides.

8. A method of Claim 4 wherein the second portion has a nucleic acid sequence comprising the nucleic acid sequence of SEQ ID NO:1.

9. A method of Claim 1 wherein the forward primer has a length in the range of from 16 nucleotides to 30 nucleotides.

10. A method of Claim 1 wherein the forward primer consists of the nucleic acid sequence set forth in SEQ ID NO:13.

11. A method of Claim 6 wherein the forward primer hybridizes to the complement of the second portion of the extension primer.

12. A method of Claim 1 wherein the forward primer comprises a locked nucleic acid molecule.

13. A method of Claim 12 wherein the forward primer comprises from 1 to 3 locked nucleic acid molecules.

14. A method of Claim 1 wherein the reverse primer has a length in the range of from 12 nucleotides to 20 nucleotides.

15. A method of Claim 1 wherein the reverse primer comprises a locked nucleic acid molecule.

16. A method of Claim 1 wherein the reverse primer comprises from 1 to 3 locked nucleic acid molecules.

17. A method of Claim 1 wherein the reverse primer is selected to specifically hybridize to a selected microRNA molecule under defined hybridization conditions.

18. A method of Claim 1 further comprising the step of measuring the amount of amplified DNA molecules.

19. A method of Claim 1 wherein amplification is achieved by multiple successive PCR reactions.

20. A method for measuring the amount of a target microRNA in a multiplicity of different cell types within a living organism, the method comprising the step of measuring the amount of a target microRNA molecule in a multiplicity of different cell types within a living organism, wherein the amount of the target microRNA molecule is measured by a method comprising the steps of:

(1) using primer extension to make a DNA molecule complementary to the target microRNA molecule isolated from a cell type of a living organism;

(2) using a forward primer and a reverse primer to amplify the DNA molecule to produce amplified DNA molecules, wherein one of the forward primer and the reverse primer comprises a locked nucleic acid molecule; and

(3) measuring the amount of the amplified DNA molecules.

ABSTRACT OF THE DISCLOSURE

In one aspect, the present invention provides methods for amplifying a microRNA molecule to produce DNA molecules. The methods each include the steps of: (a) using primer extension to make a DNA molecule that is complementary to a template microRNA molecule; and (b) using a forward primer and a reverse primer to amplify the DNA molecule to produce amplified DNA molecules, wherein one of the forward primer and the reverse primer comprises a locked nucleic acid molecule.

Title: METHODS FOR QUANTITATING SMALL RNA MOLECULES
Inventor: C.K. Raymond
Docket No.: ROSA123905

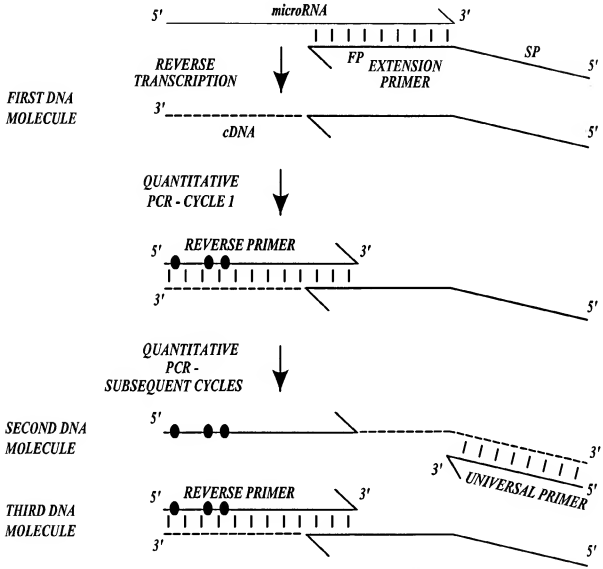

Fig. 1.

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